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Effects of Multiple Metal Resistant Bacterias on Antioxidants of *Pleurotus ostreatus* in Metal-Polluted Soil

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In this study, the effects of two heavy-metal-mobilizing bacterias CT1 and WK1 on fruiting body growth, Cd and Pb accumulation, lipid peroxidation, protein content and antioxidant system in *Pleurotus ostreatus* were assessed in Cd and Pb contaminated soil. Pot experiments showed that inoculation with CT1 and WK1 not only partly promoted the growth of *P. ostreatus*, but also enhanced the accumulation of Cd and Pb compared to control, respectively. Moreover, protein level in *P. ostreatus* inoculation with CT1 and WK1 displayed an increase. Besides, a significant decrease in malondialdehyde content suggested that both bacterial strains can alleviate lipid peroxidation in *P. ostreatus*. Antioxidant enzyme superoxide dismutase, peroxidases and catalase (CAT) activities of *P. ostreatus* also showed obviously decrease by inoculation with CT1 and WK1. Therefore, the present work suggests that heavy metal-solubilizing bacterias can reduce the toxicity of metals to fruiting body and then alleviate heavy metals-inducing oxidative stress in *P. ostreatus*.

Keywords: Heavy-metal-mobilizing bacteria, Fruiting body, *Pleurotus ostreatus*, Oxidative stress, Alleviation.

INTRODUCTION

Heavy metal pollution in soils has led to extensive environmental hazards, result in economic losses in agriculture and health problems in humans[1,2]. Especially, lead and cadmium are important and widespread pollutants and non-essential for living beings. Elevated levels of heavy metals not only decrease soil microbial activity, soil fertility and yield losses, but also induce toxicity observed in different living organisms which associated with oxidative damage[3,4]. Mushroom, as macrofungi, possess a great tolerance to high levels of heavy metal pollution, which have been well documented[5,6]. Moreover, with excessive heavy metal contamination, mushroom may initiate a variety of biochemical stress responses to protect themselves against oxidative damage, such as producing superoxide dismutase (SOD), peroxidases (POD) and catalase (CAT)[7]. Meanwhile, biochemical stress responses by using enzymatic and non enzymatic mechanisms to protect themselves from environmental stress factors have been widely reported in different living beings[8,9], especially in the case of cadmium and lead toxicity.

However, this defense system can not completely offset the damage of heavy metals for organism and the metal accumulation efficiency of mushroom is limited by the low bioavailability of metals in soils. Thus, the key problem is to find a way which not only can reduce its damage to biont, but also remove heavy metals from environment. In recent years, synthetic chemical chelators, such as EDTA, which enhance the efficiency of phytoremediation, received increasing criticism because of their potential environmental risks[10].

Recently, a promising alternative strategy, bioaugmentation has been developed to increase the bioavailability of metals in contaminated soils[11]. Because of high activity and a high surface area-to-volume ratio, special attentions have been paid on natural soil microorganisms. Bacteria and fungi which have high surface area-to-volume ratio providing a large contact area may have the potential to act as microbial chelates[12]. It has been reported that soil microorganisms can improve the metal solubility and availability through acidification[13], phosphate solubilization[14,15], redox changes[16], producing chelators[17-19] and the release of siderophores[19]. Recently, attention has been paid to induction of metal tolerance in plants and promotion of metal accumulation in plants by soil microorganisms[20,21]. However, very few literatures are available on the application of soil microorganism to alleviate metal toxicity in mushroom in soil experiment.

P. ostreatus can accumulate potentially toxic metals, have been well documented. Meanwhile, the oxidative damage

caused by heavy metals in mushroom has also been investigated[22]. But, there is no study on how to alleviate oxidative damage induced by heavy metals in the fruiting body of *P. ostreatus*. The objective of this study was to investigate the role of soil microorganism in promoting *P. ostreatus* growth and alleviating cadmium and lead toxicity in the fruiting body of *P. ostreatus* in soil experiment. The main purposes were (a) isolate two Cd and Pb multiple heavy metal-resistant and heavy metal-solubilizing strain; (b) identify two bacteria through determination of 16S rDNA gene sequences; (c) characterize two bacteria by testing indole acetic acid (IAA), phosphate solubilization and siderophore; (d) most importantly, evaluate the effects of the two strains on *P. ostreatus* biomass, heavy metals enrichment content, malondialdehyde (MDA) content, soluble protein level and antioxidant enzymes activities (superoxide dismutase, peroxidases and catalase) in Cd and Pb joint toxicity.

EXPERIMENTAL

Isolation of multiple heavy metal-resistant and heavy metal-solubilizing bacteria: Two Cd and Pb resistant strains were isolated from contaminated hyphosphere soils that were collected from a lead mine tailing in the suburbs of Deyang, China. About 1 g soil samples were serially diluted using 25 mM phosphate buffer and spread on sucrose-minimal salts low-phosphate (SLP) medium (1 % sucrose, 0.1 % $(NH_4)_2SO_4$, 0.05 % K_2HPO_4, 0.05 % $MgSO_4$, 0.01 % NaCl, 0.05 % yeast extract, pH 7.2) amended with 200 mg/L of Pb [$Pb(NO_3)_2$], 50 mg/L of Cu [$Cu(NO_3)_2$]. The plates were incubated at 37 °C for 72 h. From the resistant colonies, different strains were picked and purified on the salts low phosphate medium containing heavy metals as described above. Purified colonies were gradually taken to higher concentration of Pb (200-800 mg/L) and Cd (50-200 mg/L) using the plate dilution method[23]. Two strains CT1 and WK1 that could tolerate 800 mg/L of Pb and 200 mg/L of Cd simultaneously were selected for further study. Solubilization of insoluble heavy metals by the isolates was studied with heavy metal carbonate mobilizing test in liquid medium according to procedure of Jiang *et al*[15].

Strain identification(16S rDNA genes sequencing): The bacterial strains were identified by determination of 16S rDNA gene sequences. Bacterial cells were harvested after growing in Luria-Bertani (LB) broth for 20 h and processed immediately for genomics DNA isolation using standard procedure[24]. 16S rDNA was amplified in polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, 27 f (5'-GAGTTTGATCACTGGCTCAG-3') and 1492 r (5'-TACGGCTACCTTGTTACGACTT-3')[25]. The amplification products were purified using a DNA purification kit (Takara) and the sequencing was performed at Shanghai Invitrogen Biotech Company. The 16S rDNA sequences were then compared to similar sequences in the databases using the NCBI Blast program.

Characteristics of the bacteria

Quantification of indole acetic acid production: Indole acetic acid production was determined according to the method of Bric *et al*[26]. The isolated strains were inoculated in sucrose-minimal salts (SMS) medium (sucrose, 1 %; $(NH_4)_2SO_4$, 0.1 %; K_2HPO_4, 0.2 %; $MgSO_4$, 0.05 %; NaCl, 0.01 %; yeast extract, 0.05 %; $CaCO_3$, 0.05 %; pH 7.2) supplemented with 0.5 mg/mL of tryptophane and incubated at 30 °C for 96 h at 150 rpm. The supernatant was mixed with Salkowski's reagent. The absorbance was measured at 530 nm. The pure indole acetic acid concentration in culture medium was determined by a calibration curve of pure indole acetic acid as a standard following the linear regression analysis.

Determination of siderophore production: The production of siderophore by the tested strains was assessed using a chrome azurol S shuttle solution by the analytical method of Schwyn and Neilands[27]. The assay was calibrated by generating standard curve for samples containing 1-100 μM deferoxamine messylate.

Phosphate-solubilizing activity: The phosphate solubilization ability of the bacterial strains were screened using modified Pikovskaya's medium[28] with 0.5 % of tricalcium phosphate at 30 °C for 144 h at 200 rpm. The culture supernatants were collected by centrifugation at 8,000 rpm for 20 min. Soluble phosphate in the culture supernatant was quantified according to the method of Fiske and Subbarow[29].

Pot experiments: Natural brown soil used in the study was collected from agricultural field in Longquanyi, Chengdu. Some physicochemical properties of the soil were listed in Table-1. Soil was crushed to pass through a sieve which screen-aperture was 5 mm diameter. The sifted soil was artificially contaminated with Pb-Cd (200 + 5, 400 + 10, 800 + 15 mg/kg) as Pb $(NO_3)_2$ and Cd $(NO_3)_2$, respectively. In order to achieve equal distribution, soil was kept in a greenhouse in Sichuan University for a 10-month period before the pot experiment. The cultivate bag of pleurotus was purchased from Huike, a mushroom production site in Chengdu. Every cultivate bag of *P. ostreatus* was put in plastic pot containing 7 kg soil. Each treatment was performed in triplicate with constant humidity and stable temperature.

TABLE-1
PHYSICO-CHEMICAL PROPERTIES OF THE UNTREATED SOIL USED IN THE EXPERIMENT

Parameter	Soil value
pH	7.46 ± 0.03
Water holding capacity (%)	12.76 ± 0.61
Cation exchange capacity (cmol kg^{-1})	11.45 ± 0.26
Organic mater content (g kg^{-1})	18.64 ± 0.19
Total Pb (mg kg^{-1})	44.05 ± 2.54
Extracted Pb (mg kg^{-1})	9.31 ± 0.43
Total Cd (mg kg^{-1})	0.12 ± 0.03
Extracted Cd (mg kg^{-1})	0.05 ± 0.04

Data shown are means of triplicates ± SD

For inoculation, the selected bacterial strains were grown over night in sucrose-minimal salts medium. Bacterial cells were harvested in the exponential phase by centrifugation at 8000 rpm, 20 min. The pellets were washed twice with sterile distilled water and recentrifuged. Bacterial inoculums (10^7 CFU mL^{-1}) were prepared as described above. Bacterial suspensions (20 mL. pot^{-1}) were sprayed on the soil surface. The pot inoculated with 20 mL of sterilized deionized water was used as a control. Mushrooms were harvested once the fruit bodies unfolded and then washed with deionized water for three times.

Each fruiting body was cut into 2 halves. One half of samples were dried at 55 °C in an oven for 4 d until reaching a constant mass to determinate the dry weight and the contents of heavy metals. The other half was quick-frozen in liquid nitrogen to detect malondialdehyde, protein and antioxidant enzymes.

Heavy metals accumulation in *P. ostreatus:* The dried samples were ground and sieved to < 0.30 mm, then digested with concentrated HNO_3 and 30 % H_2O_2 (5:2, v/v) in microwave oven (40 min) according to the procedure of Tuzen *et al*[30]. After digestion, the volume of each sample was adjusted to 25 mL using deionized water. Contents of Pb and Cd in the samples were determined using flame atomic adsorption spectrometry (AAS; VARIAN, SpectrAA-220Fs).

Determination of soluble protein contents and lipid peroxidation in *P. ostreatus:* Soluble protein content in *P. ostreatus* was analyzed by method of Lowry *et al*[31], bovine serum albumin was used as standard. The lipid peroxidation was measured by reaction with thiobarbituric acid (TBA) to determine malondialdehyde (MDA) as the end-product of lipid peroxidation, according to the method of Heath and Packer[32]. The concentration of MDA was calculated by using the absorbance coefficient of 155 $mM^{-1} cm^{-1}$, results were expressed as nmol/g fresh weight (FW).

Activities of antioxidative enzymes

Extraction of enzymes: The fresh samples were quickly frozen in liquid nitrogen and grinded by a pre-cooled mortar and pestle. The homogenate was suspended in 20 mM *tris*, 1 mM EDTA buffer (pH 7.5) for 2 h at 4 °C. After that, the homogenate was centrifuged at 6,200 g for 10 min, the supernatant was centrifuged at 15,000 g for 0.5 h at 4 °C. Finally, the obtained cell-free extract was used to measure the activities of superoxide dismutase, catalase and peroxidases.

Superoxide dismutase (SOD): The activity of superoxide dismutase was assayed by the method of Beauchamp and Fridovich[33] according to measure its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. Inhibition of 50 % showed the expression of one Unit (1U) enzyme.

Catalase (CAT) : The activity of catalase was determined based on the decline in absorbance at 240 nm due to H_2O_2 reduction following the method of Beers and Sizer[34]. One enzyme activity unit was defined as absorbance changes 0.01 per minute.

Peroxidase (POD): Peroxidase (POD) activities were measured following the increase of absorbance at 470 nm due to the oxidation of guaiacol according to the method of Omran[35]. Enzymatic activities were expressed in units per mg of protein.

Data analysis: All data values were submitted to a one-way analysis of variance (ANOVA) with Duncan tests to confirm the variability of data and validity of results. Data analysis was performed with SPSS (Statistical Package for the Social Science version 18.0) for Windows. The Student-Newman-Keuls test was used for multiple comparison at $p < 0.05$ level between treatments.

RESULTS AND DISCUSSION

Screening and indentification of multiple heavy metal-resistant and heavy metal-solubilizing bacterial strains: For their best heavy metal-solubilizing abilities, two multiple heavy metal-resistant bacterial strains CT1 and WK1 were selected in this investigation (Table-2). Microorganisms isolated from natural environments contaminated with heavy metals often exhibit resistance to multiple pollutants. The bacterial strain CT1 and WK1 showed a high tolerance to heavy metals, especially to Cd and Pb. Full length (about 1,450 bp) 16S rDNA of two strains were sequenced. The closest genetic relatives according to NCBI database by BLAST analysis were shown in Table-3. The strain CT1 was Leifsonia shinshuensis and WK1 was *Bacillus cereus*. The strains had relatively high degree of 16S rDNA gene sequence similarity (99 %).

Characteristics of bacteria: Both microbial strains had the capacity to produce indole acetic acid, siderophore and solubilize inorganic phosphate (Table-3). Soil microbes protect plants against the harm of heavy metals probably for the production of indole acetic acid, siderophores and phosphate solubilization[36-38]. From Table-3, strain CT1 was considered as the better siderophore-producing (Ar/Ao = 0.31 ± 0.07) and phosphate-solubilizing one (43.141 ± 1.940 mg/mL), while strain WK1 as the higher IAA-producing one (10.076 ± 0.288 mg/mL). The two tested strains displayed a positive siderophore activity and siderophore production (Ar/Ao) varied from 0.24 to 0.90, which is similar with previous results[39].

TABLE 3
IDENTIFICATION AND CHARACTERISTICS OF THE ISOLATED STRAINS CT1 AND WK1

Strain	Siderophore production (Ar/Ao)	Indole acetic acid synthesis (mg/mL)	Phosphate solubilization (mg/mL)
CT1	0.31 ± 0.02a	6.404 ± 0.08a	43.141 ± 0.792a
WK1	0.45 ± 0.02b	10.076 ± 0.135b	26.391 ± 0.775b

Average ± standard deviation from three samples. Different letters in the same column indicate statistical difference ($p < 0.05$)

A number of studies have reported that indole acetic acid, siderophores and phosphate solubilization played an important role in protecting plant against toxicity by heavy

TABLE-2
WATER-SOLUBLE Pb(II), and Cd(II) CONCENTRATION (mg/L) AND pH VALUES UNDER LEAD, CADMIUM CARBONATE CONDITION AFTER 48 h CULTIVATION OF THE ISOLATED STRAINS CT1 AND WK1

Strain	Pb ($PbCO_3$, 2,000 mg/L)		Cd ($CdCO_3$, 2,000 mg/L)	
	pH value	Pb concentration	pH value	Cd concentration
Control	7.45 ± 0.10	0.81 ± 0.06	7.23 ± 0.11	1.75 ± 0.03
CT1	4.67 ± 0.05*	37.8 ± 0.70*	5.34 ± 0.08*	453 ± 11.53*
WK1	3.56 ± 0.07*	32.6 ± 1.08*	3.60 ± 0.03*	264 ± 3.61*

Average ± standard deviation from three samples. An asterisk (*) denotes a value significantly greater than the corresponding control value ($p < 0.05$)

metals. Phytohormones and phosphate-solubilization had been reported to improve the phytoextraction efficiency of plant by stimulating plant growth, increasing contaminant accumulation and tolerance[40]. Tripathi *et al.*[41] reported that inoculation with siderophore-producing bacteria (SPB) significantly increased growth of mung bean without showing any symptoms of heavy metals. But, the effects of indole acetic acid, siderophores and phosphate-solubilization on mushroom have been rarely carried out. In agreement these results, Cao *et al.*[42] reported that siderophores, indole acetic acid and phosphate-solubilization produced by six bacteria not only can promote the growth of *O. radicata*, but can also protect *O. radicata* against toxicity of Cd and Pb by decrease the activities of antioxidant enzyme superoxide dismutase (SOD) and peroxidases.

Effect of bacterial inoculation on *P. ostreatus* biomass and Heavy metals accumulation: Inoculation with strains CT1 and WK1 showed a dramatic increase compared to the control treatment on total metal uptake of *P. ostreatus* (Fig. 1). As shown in Fig. 1, total Cd and Pb uptakes increased from 69 to 107 % and 88 to 119 % for *P. ostreatus* with CT1 inoculation, respectively. Similarly, inoculation with WK1 also improved Cd and Pb uptakes increased from 60 to 96 % and 66 to 101 % comparing to control treatment, respectively. Compared with control, inoculation with strains Q2BJ2 and Q2BG1 was found to significantly increase the above-ground tissue (ranging from 58 to 62 %) and root (ranging from 2.1-fold to 3.5-fold) total Pb uptake[43]. Besides, Jiang *et al*[15] reported that increase in tissue Pb and Cd contents varied from 38 to 192 % and from 5 to 191 % in inoculated plants growing in heavy metal-contaminated soils compared to the control, respectively. In our study, compared to reported literatures, *P. ostreatus* inoculation with CT1 and WK1 has a significant effect on Cd and Pb uptake (Fig. 1). However, the mechanism about bacteria assisting mushrooms on solubilization, uptake and translocation of heavy metals remains unknown. Therefore, more work is still needed to be elucidated in the near future.

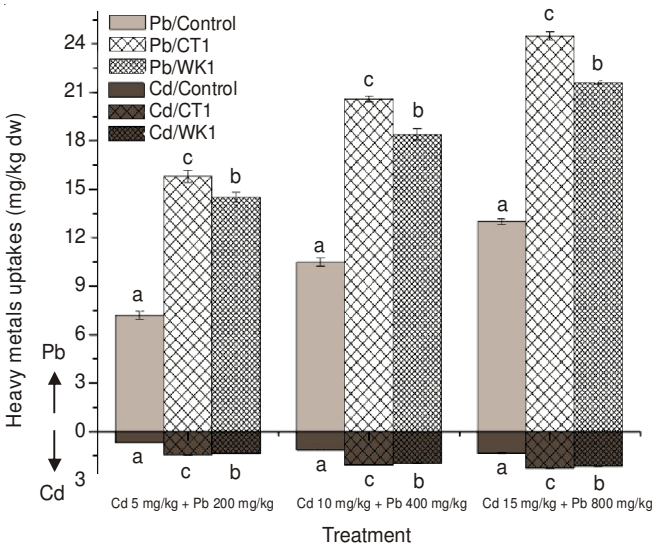

Fig. 1. Effect of bacterial inoculation on heavy metals accumulation in the fruiting body of *P. ostreatus* with different treatment. Average ± standard deviation from three samples. Different letters in the same column indicate statistical difference ($p < 0.05$)

Inoculation of CT1 and WK1 showed a dramatic enhancement on biomass of *P. ostreatus* compared with uninoculated control (Fig. 2). At low concentration (Cd 5 mg/kg + Pb 200 mg/kg), maximum growth-promoting effect was observed in CT1, Dry weight increased by 33.5 %, compared with non-inoculated *P. ostreatus*. Similarly, WK1 enhanced the dry weight by 28.2 %. In addition, at high concentration (Cd 15 mg/kg + Pb 800 mg/kg), inoculation with CT1 and WK1 were also observed an increase in dry weight by 27.6 and 20.7 %, respectively. Chen *et al.*[39] reported that at the low Cd-contaminated soil, the greatest effect was found for LSE04, of which the plants shoot length, fresh weight and dry weight enhanced by 13.7, 28.2 and 41.4 %, respectively. In this study, it was observed that inoculation with CT1 and WK1 could alleviate metal toxicity and exhibit an increase in dry weight of *P. ostreatus* compared with control. It may be attributed to the microbes to secrete some growth-stimulating substances such as indole acetic acid that stimulate fruiting body formulation. In addition, microbes reduce the metal toxicity to alleviate the growth impediment of fruit bodies imposed by the metals.

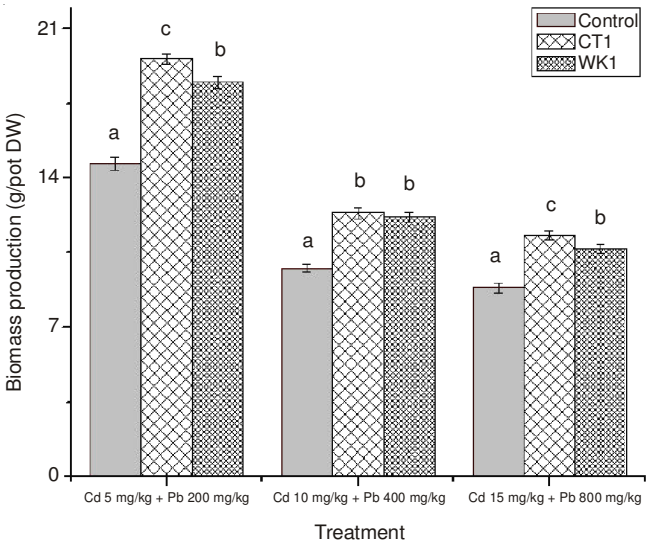

Fig. 2. Effect of bacterial inoculation on *P. ostreatus* biomass with different treatment. Average ± standard deviation from three samples. Different letters in the same column indicate statistical difference ($p < 0.05$

Lipid peroxidation products: In order to assess the potential and influence on membrane damage of CT1 and WK1, lipid peroxidation in the fruiting body of *P. ostreatus*, malondialdehyde content was measured and the values were given in Fig. 3. Either non-inoculated with CT1 or WK1, malondialdehyde contents in the fruiting body of *P. ostreatus* were significantly increased with heavy metal stress. However, malondialdehyde of inoculation with CT1 and WK1 fruiting body showed obvious decrease at three concentrations of Cd and Pb. The significant difference between uninoculation and inoculation with CT1 as well as WK1 was observed in *P. ostreatus*. Therefore, CT1 and WK1 can partly alleviate lipid peroxidation in *P. ostreatus* under metal stress. According to Dimkpa *et al.*[19], the similar result was observed that higher malondialdehyde content in control treatment (with heavy metals) than microbe treatment.

Soluble protein contents: Protein level in the fruiting body of *P. ostreatus* decreased along with the increasing concentrations of Cd and Pb, which was related with increased toxicity of heavy metals (Fig. 3). There was a significant decrease for protein content at 15 mg/kg Cd and 800 mg/kg Pb.

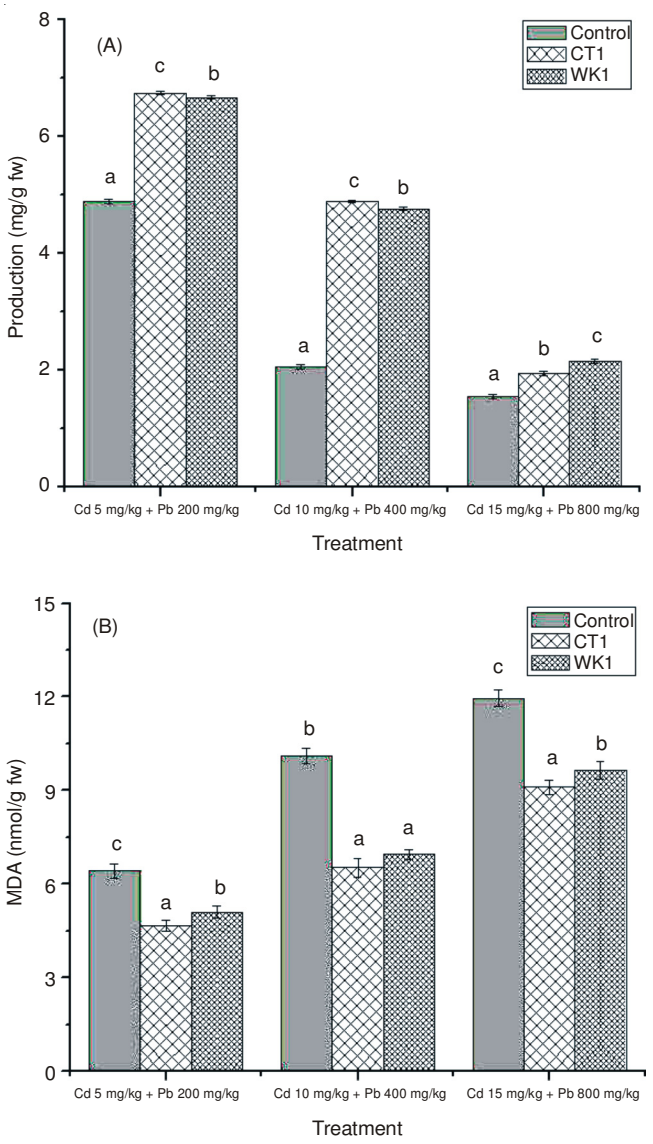

Fig. 3. Effect of bacterial inoculation on protein content in the fruiting body of *P. ostreatus* (A). Effect of bacterial inoculation on malondialdehyde (MDA) content in the fruiting body of P. Ostreatus (B). Average ± standard deviation from three samples. Different letters in the same column indicate statistical difference ($p < 0.05$)

In our study, inoculation with CT1 and WK1, protein level in the fruiting body of *P. ostreatus* showed an increase from 25.7 to 137.3 % and 36.5 to 131.3 % compared to control, respectively. Heavy metals have been shown to decrease the protein contents in the control treatment without microbes. However, protein contents in mushroom, inoculated with microbes, displayed obvious an increase[42].

Antioxidant enzymes: As the figure showed, the activities of antioxidant enzymes superoxide dismutase, peroxidases and catalase exhibited a decrease for Pb and Cd either inoculation with CT1 or WK1 compared to control (Fig. 4). The changing tendency of superoxide dismutase, peroxidases activities is

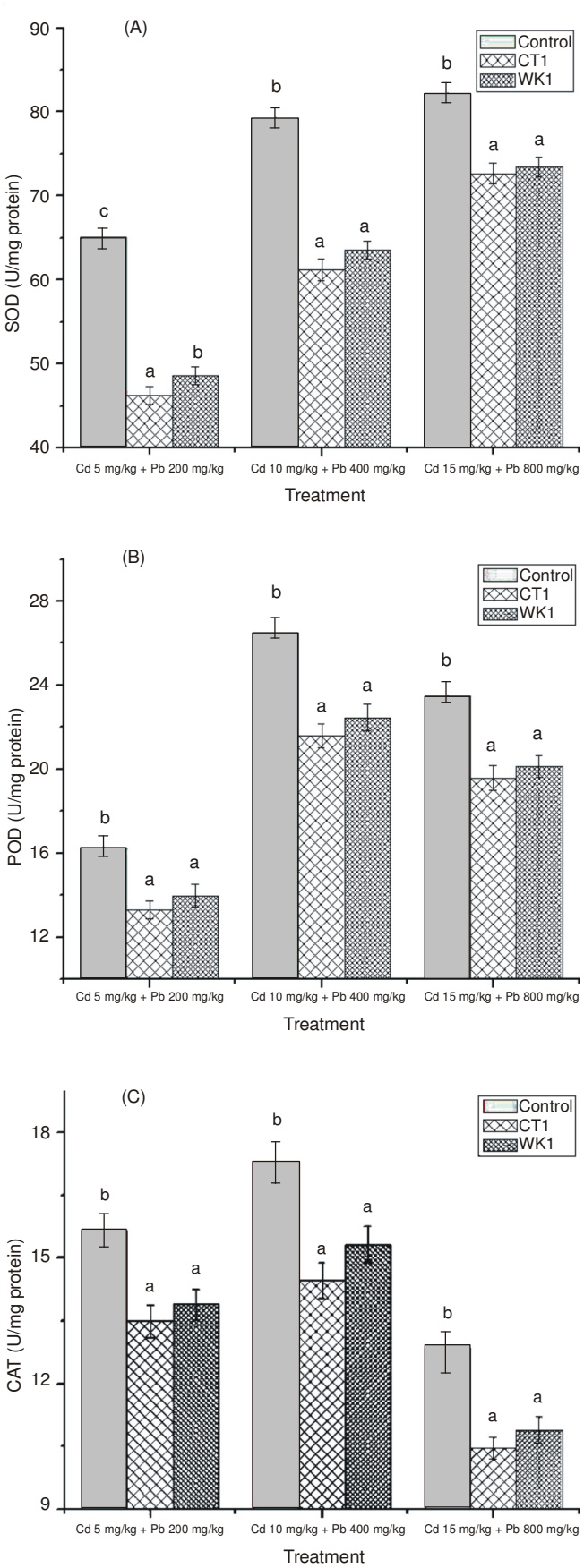

Fig. 4. Effect of bacterial inoculation on SOD activity (A), POD activity (B) and CAT activity (C) in the fruiting body of *P. ostreatus*. Average ± standard deviation from three samples. Different letters in the same column indicate statistical difference ($p < 0.05$)

analogous to catalase when *P. ostreatus* inoculation with CT1 or WK1. However, CT1 have been used successfully to alleviate oxidative stress of Cd and Pb in the fruiting body of *P. ostreatus*. Besides, superoxide dismutase activities showed a significant decrease inoculation with CT1 and WK1 in comparison to peroxidases and catalase and reached maximum at 5 mg/kg Cd and 200 mg/kg Pb (Fig. 4), decreased by 29 and 25.5 %, respectively.

As known that increased activity of antioxidant enzymes can partly reduce oxidative stress[44,45]. Exposure to heavy metals, mushrooms can respond to elevated levels of ROS by activating their antioxidative defence systems. The main enzymes involved in these defence mechanisms are the ROS-eliminating enzymes such as superoxide dismutase, catalase and peroxidases. However, in present experiment, inoculation with heavy metal-solubilizing bacterias CT1 and WK1, a significant decrease in superoxide dismutase, peroxidases (POD) and catalase activities was observed. It may be attributed that bacteria can partly alleviate metal-induced oxidative stress by lowering the formation of free radicals in fruiting body of *P. ostreatus*. Zhang *et al.*[46] reported that AM fungi have been successfully used to alleviate oxidative stress of Pb in seedlings of Zea mays. These results suggested that peroxidases, superoxide dismutase and catalase seemed to play an important role in fruiting body of *P. ostreatus*. Bacterial strains CT1 and WK1 can decrease the activities of relevant enzymes to partly alleviate the oxidative stress induced by Cd and Pb.

Conclusion

In the present work, the effect of inoculation with heavy metal-solubilizing bacteria on fruiting body growth, metals accumulation and metabolism of metal-stressed *P. ostreatus* has been mainly investigated. The conclusions can be drawn from this study as follows: Firstly, heavy metal-solubilizing bacterias CT1 and WK1 can promote the growth as well as help accumulate Cd and Pb of fruiting body of *P. ostreatus*. Secondly, the lower contents of malondialdehyde in mushroom under metal stress were detected in the inoculated treatment. The relative content of protein showed significant increase in fruiting body of *P. ostreatus* compared with non-inoculated control. Finally, all of antioxidant enzyme activities of superoxide dismutase, peroxidases and catalase displayed obviously decrease in fruiting body of *P. ostreatus* compared with non-inoculated control. From the above-mentioned results, it can be suggested that heavy metal-solubilizing bacterias played an important role in alleviating lead and cadmium joint toxicity in fruiting body of *P. ostreatus*.

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